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Revision No.: ZJ0008
Issue Date: Jul 1st, 2012

HCMV Real Time PCR Kit User Manual
For In Vitro Diagnostic Use Only

REF OD-0022-02

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For use with ABI Prism®7000/7300/7500/7900/Step One Plus; iCycler iQ™4/iQ™5; Smart Cycler II;Bio-Rad CFX 96;Rotor Gene™6000; Mx3000P/3005P;MJ-Option2/Chromo4; LightCycler®480 Instrument

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1. Intended Use

HCMV real time PCR kit is used for the detection of CMV in serum, plasma, leukocyte or urine by using real time PCR systems.

High sensitivity: lower detection line 10^3 copies/ml; LOQ: 2×10^3 ~1×10^8 copies/ml;

(Note: Analysis sensitivity depends on the sample volume, elution volume, nucleic acid extraction methods and other factors .If you use the DNA extraction buffer in the kit, the analysis sensitivity is the same as it declares. However, when the sample volume is dozens or even hundreds of times greater than elution volume by some concentrating method, it can be much higher.)

High specificity: test result will be positive, only to human cytomegalovirus.

Short operating time: 2 and a half hours totally

Good stability: kept for 12 months at –20℃; CV≤5%;

2. Principle of Real-Time PCR

The principle of the real-time detection is based on the fluorogenic 5'nuclease assay. During the PCR reaction, the DNA polymerase cleaves the probe at the 5' end and separates the reporter dye from the quencher dye only when the probe hybridizes to the target DNA. This cleavage results in the fluorescent signal generated by the cleaved reporter dye, which is monitored real-time by the PCR detection system. The PCR cycle at which an increase in the fluorescence signal is detected initially (Ct) is proportional to the amount of the specific PCR product. Monitoring the fluorescence intensities during Real Time allows the detection of the accumulating product without having to re-open the reaction tube after the amplification.

3. Product Description

Human cytomegalovirus (HCMV) is a beta human herpesvirus with a genome size of 230 Kbp, coding more then 70 viral proteins. It is a widespread opportunistic human herpesvirus that causes severe diseases in immune-compromised individuals, such as organ transplant recipients and AIDS patients.

HCMV real time PCR kit contains a specific ready-to-use system for the detection of the human cytomegalovirus by polymerase chain reaction in the real-time PCR system. The master contains reagents and enzymes for the specific amplification of HCMV DNA. Fluorescence is emitted and measured by the real time systems´optical unit. The detection of amplified HCMV DNA fragment is performed in fluorimeter **channel FAM** with the fluorescent quencher BHQ1. DNA extraction buffer is available in the kit and blood serum samples are used for DNA extraction. In addition, the kit contains a system to identify possible PCR inhibition by measuring the HEX/VIC/JOE fluorescence of the internal control (IC). An external positive control (1×10^7copies/ml) contained, allows the determination of the gene load. For further information, please refer to section 9.3 Quantitation.

4. Kit Contents

Ref.	Type of reagent	Presentation 25rxns
1	DNA Extraction Buffer	1 vial, 1.8ml
2	HCMV Reaction Mix	1 vial, 950µl
3	PCR Enzyme Mix	1 vial, 12µl
4	Molecular Grade Water	1 vial, 400µl
5	Internal Control (IC)	1 vial, 30µl
6	HCMV Positive Control (1×10^7copies/ml)	1 vial, 30µl

5. Storage

- All reagents should be stored at -20 ℃. Storage at +4 ℃ is not recommended.
- All reagents can be used until the expiration date indicated on the kit label.
- Repeated thawing and freezing (> 3x) should be avoided, as this may reduce the sensitivity of the assay.
- Cool all reagents during the working steps.
- Reaction Mix should be stored in the dark.

6. Additionally Required Materials and Devices

- Biological cabinet
- Trypsin digestive Solution
- Real time PCR reaction tubes/plates
- Pipets (0.5 µl – 1000 µl)
- Sterile microtubes
- Biohazard waste container
- Tube racks
- Desktop microcentrifuge for "eppendorf" type tubes (RCF max. 16,000 x g)
- Real time PCR system
- Vortex mixer
- Cryo-container
- Sterile filter tips for micro pipets
- Disposable gloves, powderless
- Refrigerator and Freezer

7. ⚠Warnings and Precaution

Carefully read this instruction before starting the procedure.

- For in vitro diagnostic use only.
- This assay needs to be carried out by skilled personnel.
- Clinical samples should be regarded as potentially infectious materials and should be prepared in a laminar flow hood.
- This assay needs to be run according to Good Laboratory Practice.
- Do not use the kit after its expiration date.
- Avoid repeated thawing and freezing of the reagents, this may reduce the sensitivity of the test.
- Once the reagents have been thawed, vortex and centrifuge briefly the tubes before use.
- Prepare quickly the Reaction mix on ice or in the cooling block.
- Set up two separate working areas: 1) Isolation of the RNA/ DNA and 2) Amplification/ detection of amplification products.
- Pipets, vials and other working materials should not circulate among working units.
- Use always sterile pipette tips with filters.
- Wear separate coats and gloves in each area.
- Do not pipette by mouth. Do not eat, drink, smoke in laboratory.
- Avoid aerosols

8. Sample Collection, Storage and transport

- Collect samples in sterile tubes;
- Specimens can be extracted immediately or frozen at -20 ℃ to -80 ℃.
- Transportation of clinical specimens must comply with local regulations for the transport of etiologic agents

9. Procedure

9.1 DNA-Extraction

DNA extraction buffer is supplied in the kit, please thaw the buffer thoroughly and spin down briefly in the centrifuge before use. It's better to use commercial kits for nucleic acid extraction.

9.1.1 Serum or plasma sample

1) Pipet 50µl serum or plasma to a 0.5ml tube, add 50µl DNA extraction buffer, close the tube then vortex for 10 seconds. Spin down briefly in a table centrifuge.
2) Incubate the tube for 10 minutes at 100 ℃.
3) Centrifuge the tube at 13000rpm for 10 minutes. The supernatant contains the DNA extracted and can be used for the template of the PCR.

9.1.2 Leukocyte sample

1）Isolate Leukocyte from 1ml non-heparin anticoagulant. (protocol in detail is omitted here.)
2) Take the liquid with Leukocyte into a new 1.5ml tube, centrifuge the tube at 2000rpm for 10 minutes, and carefully remove and discard supernatant from the tube without disturbing the pellet.
3) Add 50µl DNA extraction buffer, and close the tube then vortex for 10 seconds. Spin down briefly in a table centrifuge.
4) Incubate the tube for 10 minutes at 100 ℃.
5) Centrifuge the tube at 13000rpm for 5 minutes. The supernatant contains the DNA extracted and can be used for PCR template.

9.1.3 Urine sample

1) Take 1.5 ml sample to a tube, Centrifuge the tube at 13000rpm for 2 minutes, carefully remove and discard supernatant from the tube without disturbing the pellet.
2) Add 1.0ml normal saline then vortex vigorously. Centrifuge the tube at 15000rpm for 5 minutes, carefully remove and discard supernatant from the tube without disturbing the pellet.
3) Add 50µl DNA extraction buffer, close the tube then vortex for 10 seconds. Spin down briefly in a table centrifuge.
4) Incubate the tube for 10 minutes at 100 ℃.
5) Centrifuge the tube at 13000rpm for 5 minutes. The supernatant contains the DNA extracted and can be used for PCR template.

Attention:

A. During the incubation, make sure the tube is not open, for the vapor will volatilize into the air and may cause contamination in case the sample is positive.
B. The extraction sample should be used in 3 hours or stored at -20 ℃ for one month.
C. DNA extraction kits are available from various manufacturers. You may use your own extraction systems or the commercial kit based on the yield. For DNA extraction, please comply with the manufacturer's instructions.

9.2 Internal Control

It is necessary to add internal control (IC) in the reaction mix. Internal Control (IC) allows the user to determine and control the possibility of PCR inhibition.
Add the internal control (IC) 1µl/rxn and the result will be shown in the HEX/VIC/JOE.

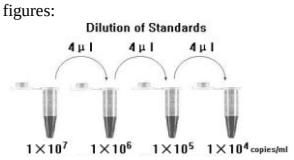
9.3 Quantitation

The kit can be used for **quantitative** or **qualitative** real-time PCR. A positive control defined as 1×10^7copies/ml is supplied in the kit.

For performance of quantitative real-time PCR, Standard dilutions must prepare first as follows. Molecular Grade Water is used for dilution.

Dilution is not needed for performance of qualitative real-time PCR.

Take positive control (1×10^7copies/ml) as the starting high standard in the first tube. Respectively pipette **36ul** of Molecular Grade Water into next three tubes. Do three dilutions as the following figures:



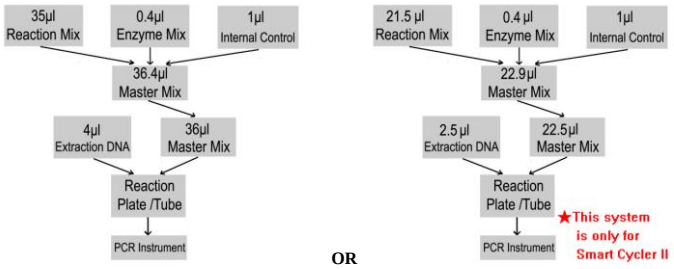
To generate a standard curve on the real-time system, all four dilution standards should be used and defined as standard with specification of the corresponding concentrations.

Attention:

A. Mix thoroughly before next transfer.
B. The positive control contains high concentration of the target DNA. Therefore, be careful during the dilution in order to avoid contamination.

9.4 PCR Protocol

The Master Mix volume for each reaction should be pipetted as follows:



※ **PCR system without HEX/VIC/JOE channel may be treated with 1µl Molecular Grade Water instead of 1µl IC.**

1) The volumes of Reaction Mix and Enzyme Mix per reaction multiply with the number of samples, which includes the number of controls, standards, and sample prepared. Molecular Grade Water is used as the negative control. For reasons of unprecise pipetting, always add an extra virtual sample. Mix completely then spin down briefly in a centrifuge.
2) Pipet **36µl (22.5µl for SmartCyclerII)** Master Mix with micropipets of sterile filter tips to each of the *Real time* PCR reaction plate/tubes. Separately add **4µl(2.5µl for SmartCyclerII)** DNA sample, positive and negative controls to different reaction plate/tubes. Immediately close the plate/tubes to avoid contamination.
3) Spin down briefly in order to collect the Master Mix in the bottom of the reaction tubes.
4) Perform the following protocol in the instrument:

37 ℃ for 2min	1cycle
94 ℃ for 2min	1cycle
93 ℃ for 15sec, 60 ℃ for 1min (Fluorescence measured at 60 ℃)	40cycles

Selection of fluorescence channels	
FAM	Target Nucleic Acid
HEX/VIC/JOE	IC

5) ⚠ If you use ABI Prism® system, please choose **"none"** as **passive reference** and **quencher.**

10. Threshold setting: just above the maximum level of molecular grade water.

11.Calibration for quantitative detection: Input each concentration of standard controls at the end of run, and a standard curve will be automatically formed.

12.Quality control: Negative control, positive control, internal control and QS curve must be performed correctly, otherwise the sample results is invalid.

Channel Control	Ct value	
	FAM	HEX/VIC/JOE
Molecular Grade Water	UNDET	25~35
Positive Control(qualitative assay)	≤35	——
QS（quantitative detection）	Correlation coefficient of QS curve≤－0.98	

13. Data Analysis and Interpretation : The following results are possible:

	Ct value		Result Analysis
	FAM	HEX/VIC/JOE	
1#	UNDET	25~35	Below the detection limit or negative
2#	≤38	——	Positive; and the software displays the quantitative value
3#	38～40	25~35	Re-test; If it is still 38~40, report as 1#
4#	UNDET	UNDET	PCR Inhibition; No diagnosis can be concluded.

For further questions or problems,please contact our technical support at **trade@liferiver.com.cn**